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REPORTS

EPINEPHRINE ACTIVATION OF PIG SKIN ADENYLATE CYCLASE IN VIVO AND SUBSEQUENT REFRACTORINESS TO ACTIVATION

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Epinephrine injected intradermally activated pig skin adenylate cyclase and increased the epidermal cyclic AMP level in vivo. This biphasic response reached a maximum in 5 min and gradually decreased thereafter. The simultaneous injection of a cyclic AMP phosphodiesterase inhibitor, isobutyl methyl xanthin (IBMX) potentiated the increase. The simultaneous injection of a specific β-adrenergic receptor inhibitor, propranolol, inhibited this accumulation of cyclic AMP. After the first activation by epinephrine in vivo, there was a marked refractoriness of the skin (epidermal) adenylate cyclase to subsequent epinephrine stimulation in vivo and in vitro. This refractoriness was specific for catecholamine stimulation as responses to histamine were normal. Recovery from refractoriness started at 48 hr and was completed at 1 week after the injection of epinephrine.

We have reported using an in vitro system that the epidermal cell possesses 4 distinct sites which can activate adenylate cyclase and result in an increase in cyclic AMP within the cell. These are: (1) the catecholamine; (2) prostaglandin (E series); (3) histamine, and (4) adenosine sites [1–4].

Using the same in vitro system we have shown that after the addition of any one of these agents a specific refractoriness developed to further stimulation by the same agent but not to stimulation by the other agents [5].

In this communication, we report the effect of epinephrine in an in vivo system. We have followed the kinetics of cyclic AMP accumulation and subsequent refractoriness to epinephrine, as well as time course of the recovery from the refractoriness in vivo.

MATERIALS AND METHODS

Domestic pigs weighing about 6 kg were anesthesized with Nembutal intraperitoneally (dose 30 mg/kg). This procedure was necessary to obtain a thin and standardized skin from a rather restricted area of the injection site. Since thiobarbiturate, a closely related compound of Nembutal, is known to increase the blood level of histamine [6], and since histamine has been shown to activate epidermal adenylate cyclase resulting in the accumulation of cyclic AMP in in vitro [3], we examined the effect of Nembutal on the cyclic AMP level of the skin. There was no accumulation of cyclic AMP in the skin up to 45 min after the injection of Nembutal at this dose (data not shown).

Fifteen minutes after the anesthesia, epinephrine hydrochloride in saline (200 μl) was injected intradermally in the back skin of the pig with a tuberculin syringe and a 25 gauge needle. As the control, saline was injected into the opposite site of the back. Usually 5 injections were made approximately 3 inches apart for each side. The epinephrine injection site was either located by the blanching of the skin which appeared immediately after the injection, or by the purpuric spots which followed several hours after the blanching and lasted for several days. Neither the blanching nor purpura was observed at the saline injection site.

After an appropriate time, the skins from both control and experimental sites were taken by a keratome set at 0.2 mm depth and was immediately frozen between 2 plates of Dry Ice for the measurement of cyclic AMP levels in vivo. For the in vitro incubation experiments (e.g. Table I) the skin pieces thus obtained by the keratome were cut into 5 × 5 mm squares and after pre-incubation in Hank's medium for 15 min at 37°C 2 of the epidermal squares were randomly selected and carefully floated with their keratin layers up on Hank's medium containing the drugs to be tested. After the incubation at 37°C, they were quickly frozen between 2 plates of Dry Ice. The cyclic AMP content was measured by Gilman's protein binding method [7] with minor modifications in the method of extracting cyclic AMP [8,9]. Protein was measured by the method of Lowry et al [10]. Epinephrine hydrochloride was obtained from Parke Davis (Detroit, Michigan). d.l. Propranolol hydrochloride and histamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

As shown in Fig 1, epinephrine caused a marked increase in the intracellular cyclic AMP level when it was injected intradermally. The maximal accumulation was observed by 5 min and gradually decreased. The simultaneous injection of phosphodiesterase inhibitor isobutyl methyl xanthin (IBMX) markedly potentiated the effect of epinephrine, and a high level of cyclic AMP was noted for at least 30 min after the injection. Fig 2 shows the effect of different concentrations of epinephrine injected intradermally. Apparently 2×10^{-2} mg (≒0.1 μmole) of epinephrine was required to give a maximal response, but 2×10^{-3} mg (0.01 μmole) of epinephrine did not show any effect at all. The effect of specific β-adrenergic antagonist propranolol is shown in Fig 3. The effect of epinephrine was significantly inhibited by the simultaneous a 10-fold greater concentration of propranolol (10 μmoles propranolol and 1 μmole epinephrine) could not block the effect of epinephrine completely. Saline injection had no effect throughout these 3 experiments.

Table I shows the effect of epinephrine and histamine in vitro on the cyclic AMP level of skin after pretreatment in vivo with epinephrine by injection. At appropriate times after the injection of epinephrine, the skin of the injected site was removed by a keratome and the cyclic AMP level of the skin was assayed after in vitro incubation with epinephrine or histamine. Both drugs were used at their saturation concentrations to cause maximum cyclic AMP accumulation in pig skin [1,3]. The injection of epinephrine caused a marked decrease in the response to a second stimulation by the same agent. Thus, 30 min after the injection, the cyclic AMP accumulation was only 60% of the initial response. After 1, 1½, 3 and 5 hr no apparent response to epinephrine was observed. The skin from the uninjected and saline injected sites responded to epinephrine almost to its initial response several hours after the injections.

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Abbreviation:
IBMX: isobutyl methyl xanthin

TABLE I. *Development of refractoriness to epinephrine injected intradermally*

	Cyclic AMP pmoles/mg protein		
	No Addition	Epinephrine	Histamine
No injection (control)	1.4	24.9	49.9
10 min after injection	1.9	21.8	41.5
30 min after injection	0.8	14.8	24.7
90 min after injection	0.3	1.5	18.5
3 hr after injection	0.3	0.5	19.6
5 hr after injection	0.3	1.0	(ND)[a]
5 hr after injection No injection (control) sites	2.0	20.4	50.8
6 hr after injection, saline (control) injection sites	1.5	20.0	(ND)[a]

Epinephrine (200 μg/200 μl) was injected intradermally and at appropriate times after the injection the skin was taken by a keratome. The response to second stimulation by the skin was determined after the in vitro incubation (see Materials and Methods). No phosphodiesterase inhibitor was added to the incubation media. Concentrations of epinephrine and histamine were 50 μM and 1 mM respectively. Each figure is an average of duplicates.

[a] ND = not determined.

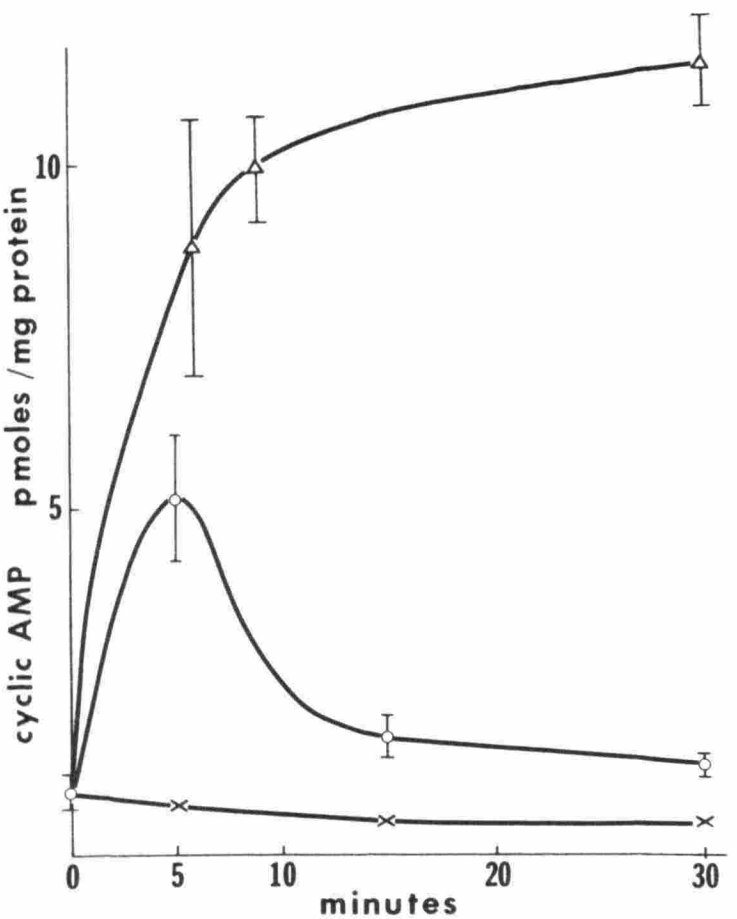

FIG 1. Time course of the effect of epinephrine on the intracellular cyclic AMP content. Detailed experimental conditions are given in the Materials and Methods section. The concentration of epinephrine was 200 μg/200 μl and that of isobutyl methyl xanthin (IBMX) 0.5 μmoles/200 μl (2.5 mM). The results of epinephrine injection are expressed as mean ± SE, n = 4 ~ 6 with 2 different pigs. Data on the control and IBMX groups are averages of duplicate determinations. ○ = epinephrine only; △ = epinephrine + IBMX; *X* = control (saline injection).

The refractoriness experiments described as above were done with an in vitro (keratome slice) system after the initial in vivo hormone stimulation. We have also tested the development of refractoriness in a completely in vivo system, i.e., the intradermal cyclic AMP levels were measured after 2 consecutive injections of epinephrine. The results of duplicate experiments are summarized in Table II. When epinephrine was injected 24 hr prior to the second epinephrine injection at the same site, the stimulatory effect of epinephrine was completely abolished. However, when saline was injected first, the epinephrine injection 24 hr later clearly increased local cyclic AMP level.

The initial epinephrine stimulation also caused a decrease in the response to histamine (Table I). After 90 min, the histamine response was about 40% of the control value, but a significant accumulation of cyclic AMP was still observable. The partial loss of response to histamine was limited to the epinephrine injected sites and the skin from the uninjected site after 4 hr responded normally to histamine.

Table III shows the effects of epinephrine and histamine in vitro after the initial in vivo epinephrine injections at 2 different doses (c.f. Fig 2). Twenty-four hours after the injection of a maximal cyclic AMP accumulating dose (10^{-2} mg), the skin no longer showed a response to epinephrine. On the contrary, skin injected with a dose (10^{-4} mg) previously shown not capable of elevating cyclic AMP in vivo subsequently responded to the second epinephrine stimulation as fully as the skin with no injection or with saline injection did. Good responses to histamine were found except for a slight decrease with the 10^{-2} mg

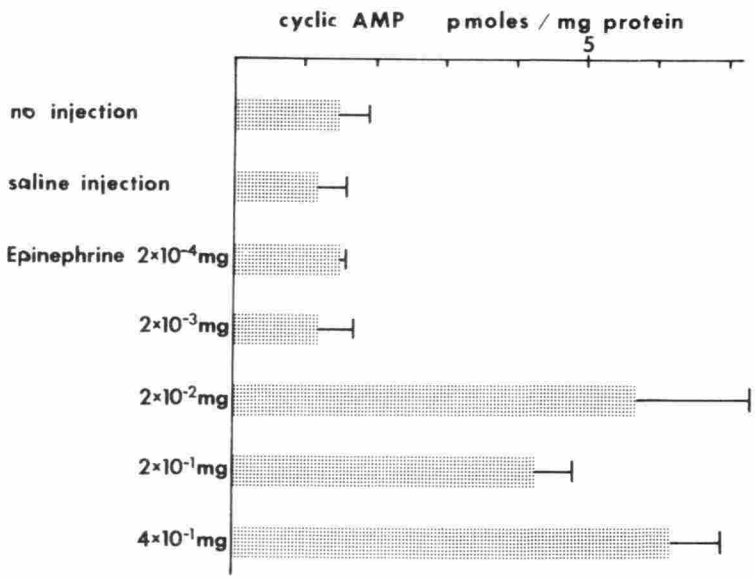

FIG 2. The effects of various concentrations of epinephrine. The skin was taken 5 min after the injection of epinephrine or saline for cyclic AMP measurements. No phosphodiesterase inhibitor was added. The results are expressed as mean ± SE, n = 4 ~ 6 with 2 different pigs.

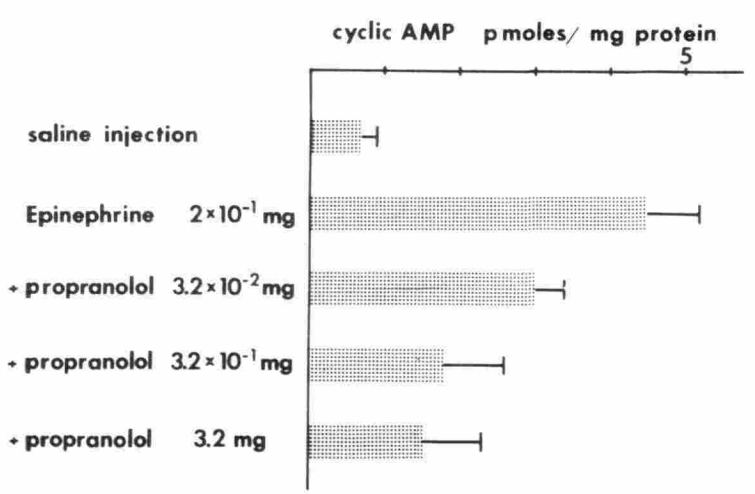

FIG 3. The effect of propranolol on the epinephrine activation. The experimental conditions were the same as in Fig 3. Propranolol was added to the epinephrine solution immediately before the injection. Results are expressed as mean ± SE, n = 4 ~ 6 with 2 different pigs.

TABLE II. *Development of refractoriness in vivo*

	Cyclic AMP pmoles/mg protein
No injection	1.8
Epinephrine injection	9.9
A) Epinephrine— Epinephrine injections	1.5
B) Saline— Epinephrine injections	7.4

The initial injection of (A) epinephrine (200 μg/200 μl) or (B) saline (200 μl) was done 24 hr prior to the second injections, which were epinephrine (200 μg/200 μl) for both the sites A and B. Five minutes after the second injection the skin samples were taken by a keratome and frozen immediately between 2 plates of Dry Ice.

TABLE III. *Development of refractoriness to epinephrine after 2 different doses of epinephrine injection*

	cyclic AMP pmoles/mg protein		
	No Addition	Epinephrine	Histamine
No injection	2.1	15.1	37.8
Saline injection	1.4	19.2	38.5
A) Epinephrine injection 2×10^{-4} mg	1.6	14.9	43.3
B) Epinephrine injection 2×10^{-1} mg	1.5	1.3	29.2

Epinephrine or saline was injected intradermally (200 μl). Twenty-four hours later the skin was removed by a keratome. Other procedures are the same as in Table I. Each figure is an average of duplicate assays.

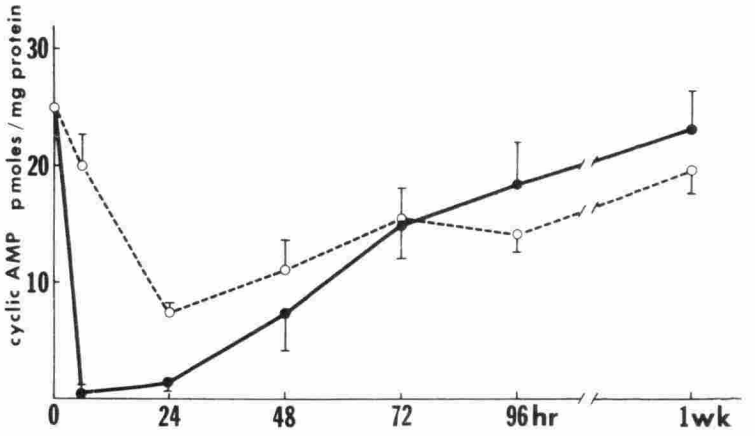

FIG 4. Recovery from the refractoriness to epinephrine. Epinephrine (200 μg/200 μl in saline) or saline (200 μl) was injected intradermally. After appropriate time, the skin from the injection sites were removed by a keratone. Changes in the hormone response was determined in an in vitro system, i.e., cyclic AMP levels of the sliced skin were determined after the in vitro incubation with epinephrine (50 μM). No phosphodiesterase inhibitor was added in the incubation media. The results are expressed as mean ± SE, n = 6 with 3 different pigs, ○ = saline injection sites; ● = epinephrine injection sites.

injection. An accumulation of cyclic AMP preceded the development of the refractoriness and amounts of epinephrine which were insufficient to produce an increase in cyclic AMP were also insufficient in producing a subsequent refractory period.

Figure 4 shows the refractoriness of pig skin to epinephrine and the recovery from the refractoriness. Again, 6 hr after the injection of epinephrine there was a complete loss of response to epinephrine at the epinephrine injected site. The refractoriness continued for at least 24 hr. Forty eight hours after the injection there was a substantial response to epinephrine. By 1 week, recovery was complete and the response to epinephrine was about the same as the initial response.

On the saline injected site there was also a decrease of epinephrine response, which was most remarkable after 24 hr. After 24 hr the response to epinephrine increased slowly with time. Both the epinephrine and saline injected sites showed refractoriness to epinephrine but the degree of refractoriness was obviously higher in the epinephrine injected site. Statistical analysis showed that the difference in the mean cyclic AMP levels in the control and epinephrine treated skin was significant at 6 hr and 24 hr ($P < .01$). After 48 hr the difference between the 2 was not significant.

DISCUSSION

The present study of cyclic AMP accumulation in pig skin by epinephrine injected in vivo can be compared with previous data with the in vitro system [1]. The time course of the response to epinephrine in vivo was essentially the same as that in the in vitro system, i.e., both showed a biphasic response with a peak in about 5 min. In both cases the specific β-adrenergic inhibitor, propranolol, markedly inhibited the cyclic AMP accumulation. In the in vivo system the maximal stimulation of cyclic AMP followed the injection of 2×10^{-2} mg (0.1 μmole) of epinephrine, and the cyclic AMP level was about 5 pmoles cyclic AMP/mg protein. If the drug, for example, was distributed in a volume of 1 cc, the concentration would be 1×10^{-4} M, which is 3 times the concentration needed in vitro. Also the maximum stimulation in vivo is less than that of the in vitro system which is around 20 pmoles cyclic AMP/mg protein [1]. These discrepancies might be explained by the diffusion of the drug in vivo. No data on tissue levels of epinephrine after intradermal injection is available.

Our data clearly indicate that in the in vivo system refractoriness to epinephrine develops as it did in our previous in vitro system [5]. In the in vitro system the refractoriness to epinephrine developed in 10 min, whereas, in the in vivo system it required 90 min to develop. Here again there is uncertainty of the epinephrine tissue concentration in vivo.

In the in vitro system the refractoriness was specific only for the inducing drug, i.e., the skin in the state of the refractoriness to epinephrine could react fully to histamine activation. On the other hand, in the in vivo system presented here (Table I and Table III) the epinephrine-refractory skin showed a partial refractoriness to histamine. The reason for this discrepancy is probably due to local histamine release following the nonspecific physical damage [12,13] caused by the intradermal injection of epinephrine in the in vivo system. In fact, we frequently observed petechiae following the blanching response after the epinephrine injection: which suggests the existence of vascular damage and the release of histamine.

The development of partial refractoriness to epinephrine in the control saline injection sites appears to be due to the systemic effect of multiple epinephrine injections. Since the plasma epinephrine level is 0.07 μg/1 [11], the injection of 1,000 μg (200 μg × 5 sites) epinephrine would be sufficient to increase the blood level. In fact, several seconds after the injection an increase in pulse rate was routinely observed. When only saline injections were made and the site was tested for epinephrine sensitivity, a full activation was observed. We also observed that a single epinephrine (200 μg) injection did not develop the refractoriness at the control saline injection site (the initial cyclic AMP level of 1.8 pmoles/mg protein increased to 9.9 pmoles/mg protein in 5 min).

The physiological significance of refractoriness remains obscure at present. It would seem that the development of refractoriness to a hormone does depend on prior exposure to a concentration of the hormone which is sufficient to increase the cyclic AMP level (Table III). Whether cyclic changes in refractoriness to epinephrine could be responsible for the diurnal variations of epidermal mitosis, for example, would depend on whether physiological levels of epinephrine ever reach a high enough level to stimulate cyclic AMP formation. The present study does not strongly support this hypothesis, since the recovery from the refractoriness required several days

rather than 24 hr. Alternatively the role of the refractoriness may be sought as a self-defense mechanism against excessive stimulation. Whatever the physiological role of the refractoriness may be, our data clearly show that one can stimulate epidermal adenylate cyclase in vivo but cannot keep the activated state for an extended period.

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